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Localization of light-inducible and tissue-specific regions of the spinach ribulose bisphosphate carboxylase/oxygenase (rubisco) activase promoter in transgenic tobacco plants

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Abstract

Deletions in the spinach rubisco activase (*Rca*) promoter in transgenic tobacco were analyzed to define the regions necessary for conferring light-inducible and tissue-specific expression. Transgenic plants were constructed with *Bal* 31 deletions of the *Rca* promoter fused to the coding region of the bacterial reporter gene β-glucuronidase (GUS). Analysis of the *Rca* deletion mutants localized the region conferring normal expression downstream from −294 relative to the *Rca* transcription start site. A second set of transgenic plants containing the cauliflower mosaic virus (CaMV) 35S enhancer fused to the 3′ end of the *Rca*/GUS constructs demonstrated the presence of a light-responsive element between −150 and −78 active in leaves. Regions 10 bp long within the light-responsive region, which included putative G box and GT elements, were removed by recombinant polymerase chain reaction. Deletion of the G box element resulted in a loss of gene expression in the leaves of transgenic tobacco, while deletion of the GT motif caused a 10–100-fold increase in expression in roots. However, site-directed mutagenesis of the GT motif resulted in expression patterns identical to the normal promoter. These experiments demonstrated that light-inducible and tissue-specific expression of the *Rca* promoter involves multiple *cis* elements proximal to the transcription start site, and that interactions between these elements are essential for regulating expression.

Introduction

Rubisco activase is a soluble chloroplast protein required for activation of ribulose bisphosphate carboxylase/oxygenase (rubisco) under physiological conditions [30]. Expression is regulated by light and is tissue-specific, with both phytochrome and blue-light receptors involved in light induction of the rubisco activase (*Rca*) gene [35]. *Rca* transcripts are expressed predominantly in leaves and were undetectable in roots [37], as observed with transcripts for other photosynthetic proteins [9, 12]. The rubisco and rubisco activase genes are coordinately expressed, with parallel accumulation of *Rca*, rubisco large subunit (*rbcL*), and rubisco small subunit (*rbcS*) mRNAs in barley leaves [37].

Comparison of *Rca* and *rbcS* sequences

revealed that the 5′-upstream region of the spinach *Rca* gene contains sequences that are homologous to *cis*-regulatory elements conserved among *rbcS* genes (Fig. 1). A G box motif, with the core sequence CACGTG, has been identified in numerous genes that respond to different stimuli and are expressed in different organs [36]. The G box element was essential for transcriptional activity in the *Arabidopsis rbcS-1A* [11], wheat early methionine (*Em*) [16], and parsley chalcone synthase (*chs*) [5] genes. GT motifs, characterized in the pea *rbcS-3A* [14] and bean *chs* [17] genes were present in both positive and negative regulatory elements in the plant genes. The conserved sequence LRF-1, overlapping the TATA box, is associated with ligh-responsive expression in *Lemna gibba* [7] and tomato *rbcS* genes [27]. In addition, the *Rca* 5′ sequences contain AT-rich regions which have been shown to enhance expression in *rbcS* and other genes [8, 29, 33]. We conducted the present study to identify the *cis*-regulatory elements in the spinach rubisco activase gene that control expression and to determine the regulatory properties shared with *rbcS* genes which may be important in the coordinate expression of rubisco and its regulator, rubisco activase.

Materials and methods

Generation of Rca/*GUS gene fusions*

Ca. 100 μg of CsCl-purified plasmid containing the spinach rubisco activase genomic clone in the vector pTZ-18U was digested with *Bal* 31 nuclease, as described by Maniatis *et al.* [26]. The promoter fragments were subcloned into M13mp18 and individual clones were sized on agarose gels. Six *Rca* promoter deletions were selected for analysis in transgenic tobacco, with endpoints relative to the start of transcription at −824, −718, −493, −408, −294, and −150. The promoter fragments were inserted into the *Bam* HI site of the binary vector pBI101 [20] upstream from the GUS-coding region.

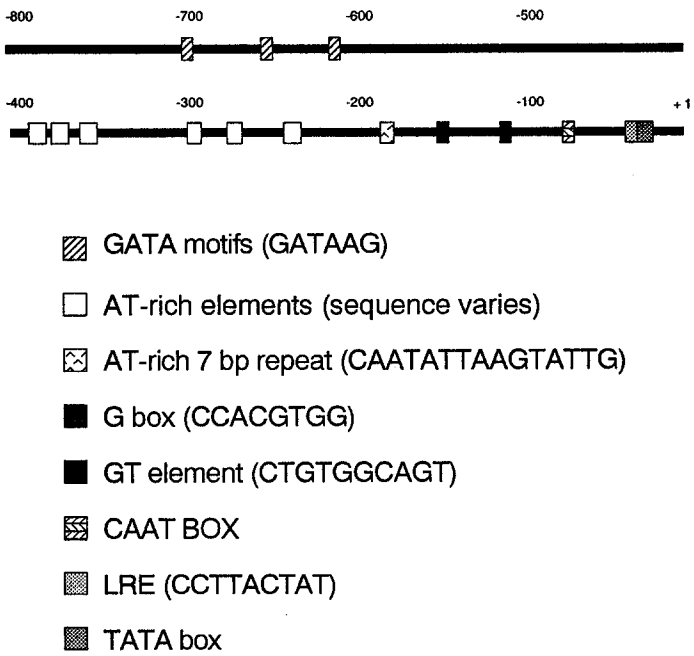

Fig. 1. Conserved DNA sequence motifs in the *Rca* promoter region. The solid black line represents the 5′-upstream region. The G box designation is from Ueda *et al.* [33], the GT element from Fluhr *et al.* [12], LRE from Grob and Stuber [15], and GATA motifs from Lam and Chua [25] and Donald and Cashmore [11].

Enhanced *Rca* promoter constructs were also generated for three of the shorter deletions. The cauliflower mosaic virus (CaMV) 35s enhancer (−343 to −90 was amplified from the promoter and fused 5′ to the GUS-coding region and nopaline synthase (NOS) terminator. The −236, −150, and −78 *Rca* promoters were inserted between the 35S enhancer and GUS-coding region. The 35S enhancer was also transferred downstream from the GUS/NOS fusion to generate 3′ enhanced promoter constructs with the −150 and −78 *Rca* fragments. Enhanced gene fusions were then subcloned into the binary vector pCGN1547 [4].

Internal deletions in the −294 rubisco activase promoter were created using PCR amplification [18]. Four sites were targeted to remove potential regulatory elements at a conserved G box, GT site, and two AT-rich sequences. Two base pair changes were generated across the putative GT element by site-directed mutagenesis using polymerase chain reaction (PCR) amplification. The modified *Rca* promoters were fused to the GUS-coding region and the gene fusions were then inserted into the binary vector pCGN1547 [4].

Agrobacterium-*mediated transformation*

Binary plasmids containing the chimeric genes were transformed into either *Agrobacterium tumefaciens* strain LBA4404 [4] or PC2760 [1] as described by Holsters *et al.* [19]. The constructs were introduced into tobacco plants via *Agrobacterium*-mediated transformation [31]. *Rca* promoter regions were amplified by PCR to confirm transformation with the *Rca*/GUS constructs in regenerated plants. Minipreparations of genomic DNA were performed with leaf punches (about 50 mg) using the methods described by Dellaporta *et al.* [10] except that the volumes were reduced 10-fold to fit into microcentrifuge tubes. The rubisco activase promoter fragments were amplified from 5 μl of the genomic DNA using primers flanking the rubisco activase promoter sequence.

Assays for GUS activity in regenerated plants

GUS activity was assayed in leaf and root extracts of transgenic plants following the methods described by Jefferson *et al.* [20]. The fluorogenic reaction was performed in extraction buffer containing 1 mM 4-methylumbelliferyl glucuronide (MUG) and 10–100 μl of extract in a final volume of 2 ml. The conversion of MUG to 4-methylumbelliferone (MU) was monitored by measuring the fluorescence for 3–10 min on a chart recorder with excitation at 365 nm and fluorescence at 455 nm. Plant extracts with GUS activity less than 100 pmol MU per minute per mg protein were incubated for 1–2 h in a 37 °C water bath and 200 μl aliquots removed at 10–15 min intervals. The reaction was stopped by mixing the aliquots with 1.8 ml of 0.2 M Na_2Co_3 and fluorescence was measured as above. The fluorimeter was calibrated with MU standards of 100, 250, and 500 μM in the same buffer. Protein concentrations were measured by the dye-binding method described by Bradford [6] with a kit supplied by BioRad Laboratories.

Northern blot analysis

Leaves of plants containing the promoters with internal deletions were harvested after 3–4 h illumination from plants grown under a 12-h photoperiod at 300 μmol photons/m^2.s and 22 °C. Dark control plants were sampled after 3 h dark adaptation. For all other constructs, leaves were harvested from plants grown under the 12 h photoperiod, incubated for 72 h in the dark, and returned to the light for 24 h. RNA was extracted with hot phenol [34]. Ca. 10 μg of total RNA was fractionated on 1% agarose gels containing formaldehyde, stained lightly with ethidium bromide to visualize the RNA, and rinsed 30–60 min in water with shaking [26]. RNA was transferred to nylon membranes by capillary blot in 10× SSC buffer (1.5 M NaCl/0.15 M sodium citrate pH 7.0) overnight and crosslinked with a UV Stratalinker (Stratagene). Single-stranded ^{32}P-labeled probes were generated by PCR amplifi-

cation of the GUS-coding region [2]. Unincorporated nucleotides were removed with Ultrafree-MC polysulfone filters (Millipore). The membranes were hybridized with the GUS probe following standard procedures [26].

Gel shift assays

Nuclear extracts were prepared from young spinach leaves and gel shift assays were performed essentially as described by Green *et al.* [14]. Binding reactions contained 1–4 ng of labeled DNA probe, 4 μg poly(dIdC)·(dIdC), 25 mM Hepes (pH 7.6), 50 mM KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 5% v/v glycerol, and 0.2–0.8 μg nuclear protein extract. All components except the labeled DNA were preincubated for 5 min to complex nonspecific DNA binding proteins and poly-dIdC. After addition of the labeled DNA, reactions were incubated for 10 min at room temperature then loaded onto 5% acrylamide gels in 0.5 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). After electrophoresis, the gels were dried onto Whatman DE81 paper and autoradiographed.

Results

Analysis of 5′ deletions of the Rca *promoter*

Constructs with 5′ deletions in the *Rca* promoter were fused to the GUS-coding region and analyzed in transgenic tobacco plants. The transgenic plants were confirmed by PCR amplification of the *Rca* promoters from genomic DNA. Southern blot analysis of representative plants for each construct (data not shown) demonstrated that the differences in GUS activity for independent transformants were not correlated to copy number. Frequently, single-copy transformants expressed higher activity than plants with multiple copies of the same construct, indicating that position effects played a significant role in the variation observed.

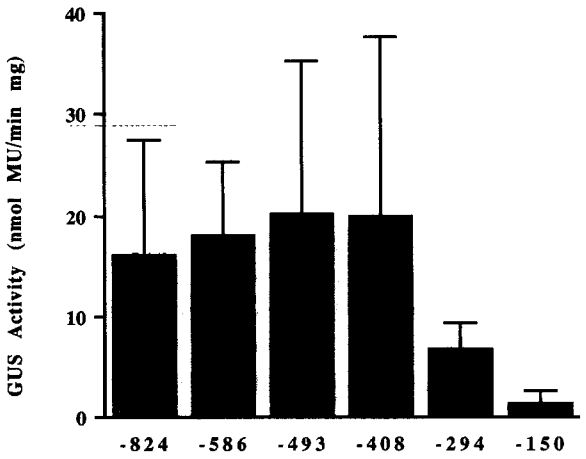

Fig. 2. GUS activity with *Rca* 5′ deletion promoters in leaves of transgenic tobacco plants. Averages from 7–12 plants for each construct are shown. Promoter sizes are designated by the 5′ end from the transcription start site. GUS activity is expressed as nmol 4-methylumbelliferone produced per minute per mg protein, with 4-methylumbelliferyl glucuronide as substrate [20].

GUS activity was measured in leaf extracts from seven to twelve transgenic plants per construct (Fig. 2). GUS enzyme activity was comparable for plants containing *Rca* promoter deletions from −824 to −408. However, a reduction in activity was observed for plants containing the −294 *Rca* promoter and activity was further reduced approximately 10–20-fold in plants containing the −150 *Rca* promoter. No GUS activity was observed in the roots of plants containing the −294 and −150 Rca/GUS constructs (data not shown). These results suggested that sequences downstream from −294 were sufficient to confer normal expression from the *Rca* promoter, although maximum expression levels may also require sequences further upstream.

Analysis of enhanced Rca *promoter constructs*

For further analysis of regulatory elements between −150 and +45, the CaMV 35S enhancer was fused to shorter *Rca* promoters to increase expression levels. As reported previously [21, 24],

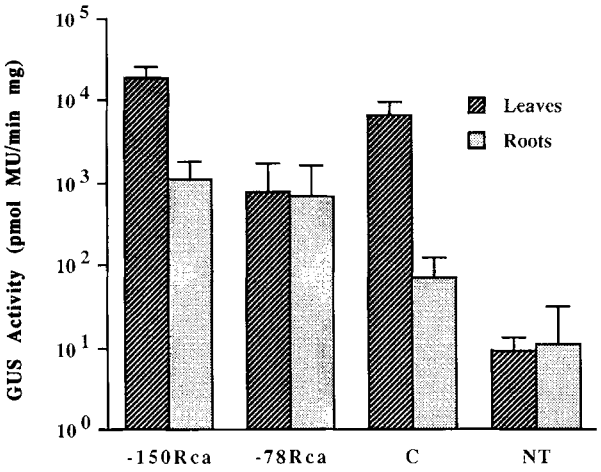

Fig. 3. GUS activity with 3′ enhanced promoters in leaves and roots of transgenic tobacco plants. Averages from 4–8 plants for each construct are shown. *Rca* promoters are designated by the 5′ end from the transcription start site. The unenhanced −294 (C) and untransformed (NT) plants are also shown. GUS activity is expressed as pmol 4-methylumbelliferone produced per minute per mg protein.

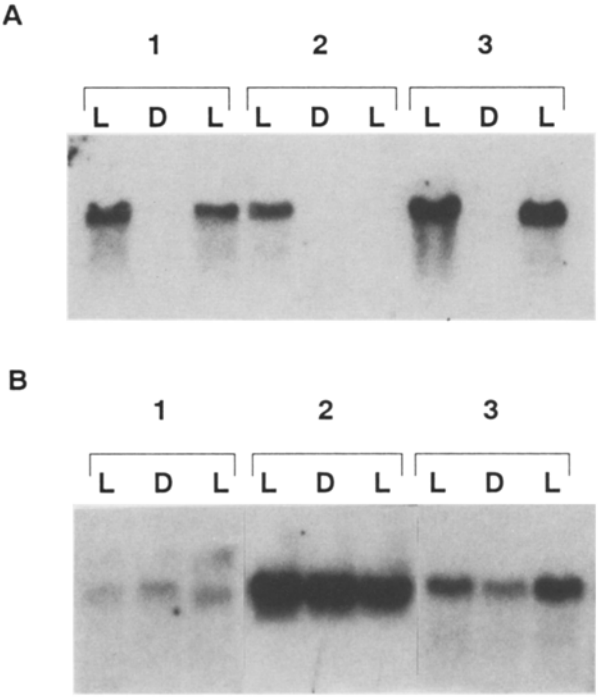

Fig. 4. Northern blot analysis of transgenic tobacco containing the 3′ enhanced −78 and −150 *Rca*/GUS gene fusions. RNA was extracted from leaves of three plants (1, 2, 3) per construct grown in the light, after 72 h continuous dark, and after 24 h return to light (shown as L, D, L). Ca. 10 μg of RNA was loaded in each lane and probed with a ^{32}P-labelled cDNA of the GUS-coding region. A. Plants containing the −150 Rca/GUS/35S construct. RNA could be seen more readily in plant 2, return to light, after longer exposure times. B. Plants containing the −78 Rca/GUS/35S construct. *Rca* promoter (Rca), β-glucuronidase (GUS), CaMV 35S enhancer (35S).

constructs with the 35S enhancer fused 5′ to the truncated *Rca* promoters were constitutively expressed. Therefore, the enhancer was inserted 3′ to the *Rca*/GUS gene fusions and tobacco was transformed with the constructs. The 3′-enhanced *Rca* promoters increased GUS expression in roots and leaves of transgenic plants compared to the unenhanced promoter fragments (Fig. 3). Northern blot analysis showed that GUS expression was attenuated in the dark for plants containing the −150 *Rca* promoter, while plants with the −78 *Rca* promoter expressed GUS constitutively (Fig. 4)

Analysis of internal deletions

Internal deletions were generated in the −294 *Rca* promoter to assess the role of potential regulatory sites in light-responsiveness, tissue specificity, and expression levels. Deletion of the AT-rich sequences −253/−275 and −168/−178 from the *Rca* promoter had no effect on expression in transgenic plants. However, analysis of transgenic plants containing the promoter with a deletion in the putative G box indicated this region may be required for activation of expression in leaves. Deletion of the G box reduced GUS activity to background levels in leaf extracts and activity in roots remained low, as observed with the normal −294 promoter (Fig. 5). No detectable GUS mRNA was visible with this construct in the Northern blot analysis (Fig. 6). Deletion of the GT motif revealed silencer activity present in the normal promoter. GUS activity increased 100-fold in roots while no change in leaf GUS activity was observed compared to the normal promoter. GUS mRNA levels expressed in leaves of light-

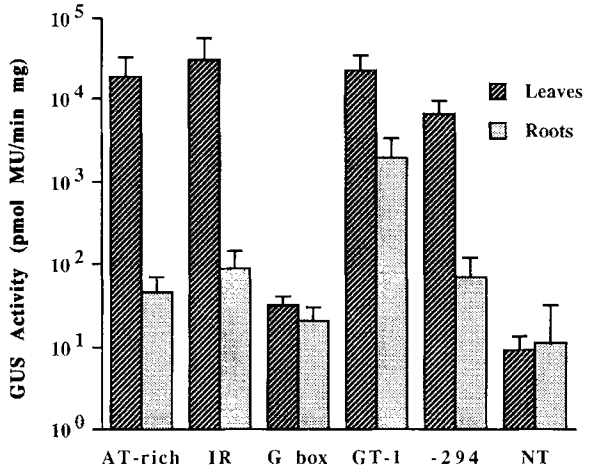

Fig. 5. GUS activity with internal deletion promoters in leaves and roots of transgenic tobacco plants. Averages from 5–7 plants are shown for each construct. Designations are −253/−275 deletion (AT-rich), −165/−178 deletion (IR), −132/−141 deletion (G box), −104/−113 deletion (GT), normal −294 *Rca* promoter (−294), and untransformed (NT).

grown and dark-adapted plants were also unaffected by the GT-site mutation. Figure 5 shows that GUS mRNA levels were high in the light and decreased after 2 d dark adaption. Two base pair mutations across the GT motif in the −294 *Rca* promoter had no effect on expression in the roots compared to the normal −294 *Rca* promoter (data not shown). Therefore, deletion of the 10 bp region apparently altered the position of adjacent regulatory elements required for tissue-specific expression rather than removing an essential sequence.

Competition experiments with gel shift assays demonstrated that spinach nuclear proteins bind specifically with the G box motif (Fig. 7). The promoter fragment from −178 to −82 formed a complex with nuclear proteins which produced at least three bands when separated on 5% acrylamide. The G box deletion promoter could not compete for the binding activity producing any of the bands, while the normal and GT deletion promoters competed for all bands. The binding activity in leaf nuclear extracts for this promoter fragment can therefore be attributed to interactions with the G box element.

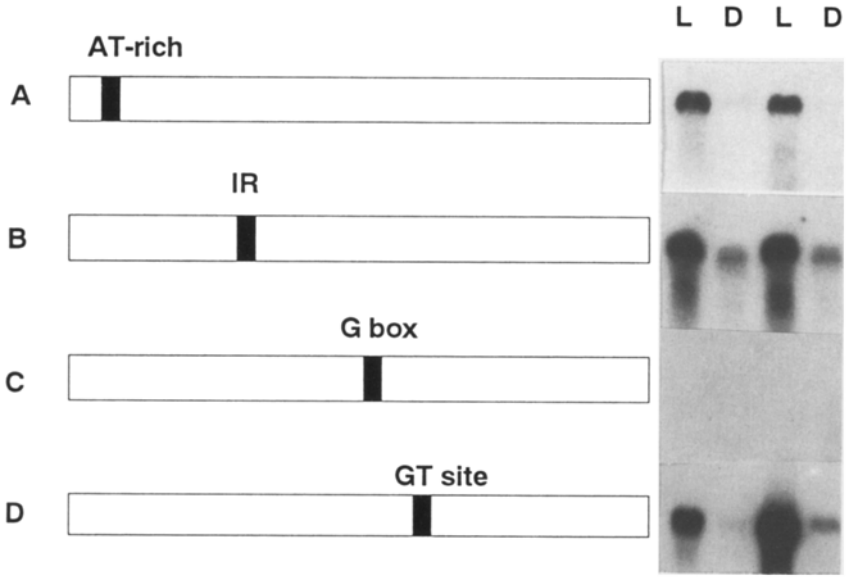

Fig. 6. Northern blot analysis of transgenic tobacco plants containing the internally deleted *Rca* promoters fused with GUS. RNA was isolated from leaves of plants grown in the light and after 48 h continuous dark. Ca. 10 μg of RNA was loaded per lane and hybridized with a ^{32}P-labelled probe of the GUS-coding region. Analyses of two plants for each construct, corresponding to the two pairs of L and D lanes, are shown. Open boxes represent the −294 *Rca* promoter and black boxes designate the location of deletion sites (described in Fig. 5).

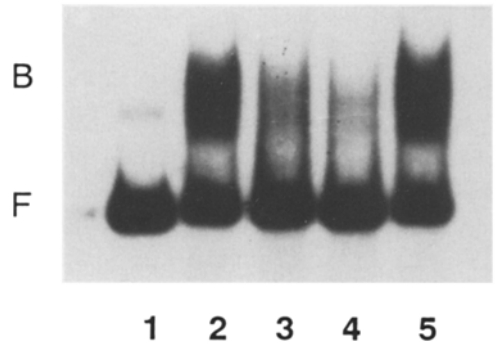

Fig. 7. Gel shift analysis with the −230 to −83 *Rca* promoter. Assay conditions included 1.6 μg spinach leaf nuclear extract protein, 4 μg poly(dIdC)·(dIdC), 2 ng of ^{32}P-labelled −230/−83 promoter fragment, and 20 ng of the unlabelled competitors. Lane 1: no extract. Lanes 2–6: plus extract. Competitors: lanes 1 and 2, none; lane 3, *Rca* promoter with IR deletion; lane 4, *Rca* promoter with GT motif deletion; lane 5, *Rca* promoter with G box deletion.

Discussion

Determination of light-regulatory regions of the Rca *promoter*

The effects of 5′ deletions in the spinach rubisco activase promoter on expression of the reporter gene GUS were analyzed in transgenic tobacco plants in order to define the regions involved in expression. GUS enzyme activity remained high in leaves of plants containing constructs with promoter deletions down to position −408 from the transcription start site. Deletion of the promoter to positions −294 and −150 decreased GUS activity in leaves, indicating that sequences between −408 and −150 were required for maximal expression. Rubisco activase is not normally expressed in roots and no GUS activity was detected in roots of plants containing the −294 or −150 *Rca*/GUS constructs. These data suggest that sequences downstream from −294 are sufficient to regulate normal expression of the rubisco activase promoter and that the region downstream from −150 confers tissue specificity. Although sequences upstream from −294 may contain functionally redundant regulatory elements [3] or enhance expression levels, they were not essential for regulation under the conditions examined. Therefore, further analysis to determine the *cis* elements controlling light regulation and tissue specificity focused on the region between −294 and the transcription start site.

Since GUS expression was reduced by deletion of the *Rca* promoter to −150 and possibly further decreased by deletion to −78, the CaMV 35S enhancer was added to the −150 and −78 *Rca*/GUS constructs to increase expression levels from these truncated promoters. The enhanced −150 *Rca* promoter conferred light-regulated expression of the GUS-coding region in leaves. Two transformants containing the −78 *Rca* promoter showed equal levels of GUS message in the light and the dark while a third plant expressed lower levels in the dark. However, GUS mRNA produced from the −78 Rca/GUS construct was reduced by about half in the dark. In contrast, a 10–20-fold decrease in GUS mRNA in the dark was observed for plants containing the −150 *Rca*/GUS constructs. These results suggest that the −78 promoter is either unable to confer light-regulated expression or contains a light-regulatory element but requires sequences upstream from −78 for full induction in the light. Since light-responsive expression was observed with the −150 *Rca* promoter, the region from −150 to −78 must confer essential regulatory properties.

Studies with the *Arabidopsis rbcS-1A* [11], wheat *Em* [28], and parsley *chs* [5] genes have shown that a G box and at least one other element conferred inducible expression. In each of these genes, the G box and the second element were located within 13 to 22 bp apart. A conserved GT motif, present in numerous viral promoters [32] and *rbcS* genes of higher plants [22], is located 21 bp downstream from the G box in the *Rca* promoter. Therefore, a deletion across the GT motif of the *Rca* promoter was analyzed in transgenic plants.

The G box is required for Rca *expression*

The 5′ deletion analysis of the *Rca* promoter defined general regions required for regulating ex-

pression. In addition, small internal deletions were generated in the −294 *Rca* promoter to assess the role of specific conserved sequences in light-responsiveness, tissue specificity, and expression levels. The −294 promoter was selected since this segment contains all the information necessary to confer normal expression while reducing the possibility of upstream regulatory elements interfering with deletion analysis. Redundancy in light regulation was detected in the pea *rbcS-3A* gene when mutations that effected expression in a truncated promoter had no effect in a longer promoter [23]. These experiments lead to the identification of upstream sequences which bind the same nuclear factor and confer light-responsive expression. The *Rca* promoter may also contain regulatory elements far upstream for controlling expression at different developmental stages and/or under different light treatments.

A conserved G box motif in the *Rca* promoter was identified as a potential regulatory element. Analysis of transgenic plants containing a 10 bp deletion across the G box in the −294 *Rca* promoter indicated that the element is necessary for activation in leaves. Deletion of the G box reduced GUS activity to background levels in leaf extracts and activity in roots remained low, as observed with the normal −294 promoter. No detectible GUS mRNA was visible with this construct in Northern blot analysis. Whether the G box is involved in light regulation was undetermined since deletion caused a complete loss of expression. However, results with the 5′ deletion analysis indicated that the promoter region containing the G box is required for light regulation. Furthermore, the promoter fragment from −178 to −82 formed a complex with leaf nuclear proteins characteristic for G boxes with an identical sequence to the motif in the *Rca* promoter [36]. The G box deletion promoter could not compete for binding activity with the normal promoter in gel shift assays. These results support the conclusion that binding of a positive-acting nuclear factor to the G box motif is required for transcriptional activity from the *Rca* promoter.

Tissue specificity is affected by a 10 bp deletion

Studies with the *Arabidopsis rbcS-1A* [11], wheat *Em* [28], and parsley *chs* [5] genes have shown that a G box and at least one other element conferred inducible expression. In each of these genes, the G box and the second element were located within 13 to 22 bp apart. A conserved GT motif, present in numerous viral promoters [32] and *rbcS* genes of higher plants [22], is located 21 bp downstream from the G box in the *Rca* promoter. Therefore, a deletion across the GT motif of the *Rca* promoter was analyzed in transgenic plants.

Light-regulated expression from the −294 *Rca* promoter was unaffected by deletion of the GT motif. However, the deletion revealed a silencer element present in the normal promoter. GUS activity increased 100-fold in roots while no change was observed for leaf GUS activity compared to the normal promoter. These results are similar to the findings of Harrison *et al.* [17] which showed that GT elements in a chalcone synthase gene could function as a negative regulatory element in roots. No protein binding activity for the *Rca* GT motif was detected with leaf nuclear extracts in gel shift assays. Deletion analysis presents two possibilities for the changes observed in root expression. Either the putative GT element may be responsible for silencing expression in the roots or the 10 bp deletion disrupted the orientation and/or distance between nearby regulatory elements. In order to address this question, 2 bp mutations were generated within the conserved GT sequence. Expression conferred by these modified promoters was unchanged compared to the normal promoter. These results demonstrated that the putative GT element is not required for regulating expression. Similarly, Gilmartin *et al.* [13] and Block *et al.* [5] showed that deletion or addition of nucleotides between regulatory sites in the pea *rbcS-3A* and parsley *chs* genes reduced or abolished promoter activity. Further analysis by DNA footprinting is required to determine the precise location of light-regulatory and tissue-specific elements of the *Rca* promoter.

Although deletion of the GT motif did not specifically locate the site of tissue-specific regulation, these results demonstrated that tissue specificity and light regulation are separable functions in the *Rca* promoter. In most cases *rbcS* promoter fragments that are light-regulated are also tissue-specific. However, a chimeric gene containing a heat shock enhancer fused to the $-50/+15$ *rbcS-3A* promoter conferred high levels of expression in leaves and roots, but the expression in leaves was light-regulated [24]. A longer *rbcS* promoter fragment fused with the heat shock enhancer was both light-regulated and tissue-specific.

Comparison of rbcS *and* Rca *gene regulation*

The arrangement of conserved sequences in the *Rca* promoter appears to determine their role in regulating expression. As shown in earlier studies with *rbcS* genes [13], the distance between *cis* elements influenced *Rca* promoter activity and interaction between multiple DNA-binding sites was required for light-regulated expression. Also, sequences in the *Rca* promoter that are homologous to known regulatory elements in *rbcS* and other genes did not necessarily confer the same regulatory properties [11, 17, 25]. These experiments demonstrate that, although the *Rca* and *rbcS* genes may share some common regulatory elements, the coordination of expression involves sequences unique to each promoter.

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